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Determination of serum alcohol using a disposable biosensor

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Abstract

This paper presents a disposable biosensor to detect serum alcohol concentration. The proposed biosensor is fabricated using the cross-linking method to immobilize Alcohol Dehydrogenase (ADH) and Nicotinamide Adenine Dinucleotide (NAD^+) on the screen-printed electrode modified with Meldola's Blue (MB) absorbed on Nafion. It is based on the electrocatalytic properties of MB as an electron transfer mediator, which can catalyze the oxidation of NADH to NAD^+ at a low oxidizing potential, thus avoiding interferences due to the presence of oxidizable substances in the real serum samples. The biosensor response for alcohol is investigated in terms of pH, buffer solution, temperature and some interferents. It presents the good specificity, reproducibility, stability, accuracy and provides a fast response. The biosensor has been satisfactorily used for the measurement of serum alcohol.

Keywords: Biosensor; Serum alcohol determination; Drunk driving

1. Introduction

Blood alcohol determination plays an important role in the forensic medicine and laboratory medicine for several reasons. First drunk driving is a very serious problem in the modern world, which results in lots of fatal accidents every year [1–5]. Blood alcohol concentration values served as the ''gold standard'' in which drivers were recognized as drunk driving by police officers [6,7]. It is also necessary for clinical laboratories to detect the acute alcoholism and some syndromes related alcohol abuse. Nowadays, many methods have been used for alcohol measurement, such as spectrometric and chromatographic analysis or breathalyzer where the alcohol concentration or refractivity was detected [8–11]. However, these methods are time consuming and complex to perform laborious sample pre-treatment. In addition, the expensive analytical apparatus is necessary. Thus, there is an increasing requirement for rapid, accurate and inexpensive methods for blood alcohol determination.

The disposable amperometric biosensor should be readily applicable to blood alcohol determination, since biosensors have such favorable analytical characteristics as portability, low cost and potential for fabrication. Several alcohol biosensors have been reported to control the fermentation process in the food and beverages industries, based on immobilization of alcohol oxidase (AOD) [12–14] or alcohol dehydrogenase (ADH) [15–18]. In an AOD-based biosensor, O_2 consumption or H_2O_2 production is determined. However, this kind of biosensor is oxygen dependent and low selectivity for alcohol. Concerning ADH-based biosensor, it is possible to directly detect reduced form of nicotinamide adenine dinucleotide (NADH) produced in the enzymatic reaction of alcohol with Nicotinamide Adenine Dinucleotide (NAD^+) under catalysis of ADH. However, the electrochemical oxidation of NADH

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involves the use of a high overpotential at which a few oxidizable substances in the real samples would be oxidized, thus increasing the likelihood of interferences. For this purpose many electron transfer mediators have been used for electrochemical oxidation of NADH, which could be detected at a low oxidizing potential [19–23]. Among them, Meldola's Blue (MB) shows some of the most promising characteristics [24,25]. However, it has been found that MB could not be absorbed stably on the electrode, meaning that the electrode is unstable for practical applications [26,27]. To improve MB attachment to the electrode, a few methods have been used [23,25]. However, these methods are complicated. Nafion, a kind of ion exchanger, can absorb MB through ion exchange [27,28]. This paper reports the immobilization of MB onto a Nafion modified screen-printed electrode (SPE).

Screen printing technology, due to its low cost and mass production, is a versatile tool for the inexpensive, easy and highly reproducible production of disposable biosensors. Up to now, most of disposable biosensors based on screen-printing technology are applied in the forensic medicine and laboratory diagnosis, since it could resolve such critical problems as low cost, fast response and portability [29].

It is the most important consideration for preparing a biosensor to easily immobilize the enzyme onto the electrode and chronically maintain its activity. This paper describes the development of disposable serum alcohol biosensor based on the cross-linking method to immobilize ADH and NAD^+ on the Nafion–MB modified SPE. The biosensor response for alcohol is investigated in terms of pH, buffer solution, temperature and some interferents. It presents the good specificity, reproducibility, stability, accuracy and provides a fast response. The biosensor has been satisfactorily used for the measurement of serum alcohol.

2. Materials and methods

2.1. Reagents

ADH (EC 1.1.1.1) NAD^+, Nafion-117 solution, MB, Glutaraldehyde are purchased from Sigma–Aldrich. Bovine serum albumin (BSA) is purchased from Roche. Carbon, sliver and polyvinyl chloride (PVC) ink are purchased from Acheson. Reference materials of alcohol are manually prepared with absolute ethanol and all of the solutions are prepared with ultrapure water from Millipore Milli-Q system.

2.2. Apparatus

A μAutolab III electrochemical analyzer (Ecochemie, Netherlands), a GC-9A gas chromatography (Shimadzu, Japan), a UV-265 ultraviolet–visible spectrophotometry (Shimadzu, Japan) and a YKP manual screen-printer (Shengjiang, GuangDong, China) are used.

2.3. Preparation of screen-printed electrode

A polyvinyl chloride film is selected as the support of SPE. The three production steps of the transducer are shown schematically in Fig. 1. The biosensor comprises a 3-mm diameter working area, the insulation layer and an electrical contact site. The biosensors are printed onto the PVC sheet. The different layers including silver conducting basal track, carbon working area and insulation layer are printed one after the other. After every step the film is left to dry for 2 h in an oven at 40 °C to drive off the solvents from the applied ink. Prepared electrodes have to be aged for 7 days at room temperature.

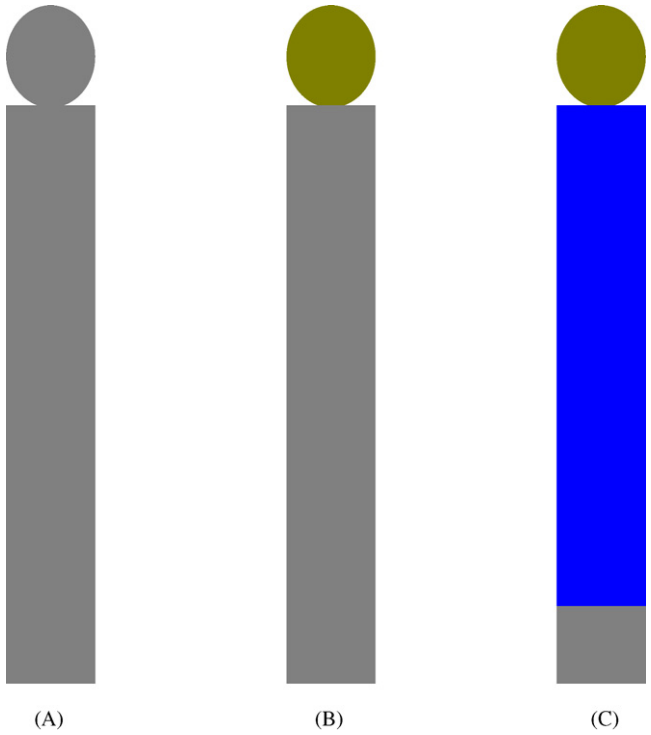

Fig. 1. Production steps for SPE. (A) Printing of sliver conducting basal track; (B) Printing of carbon working area; (C) Printing of insulation layer.

2.4. Preparation of Nafion–MB modified electrode

The SPE is ultrasonically cleaned with ethanol and ultrapure water for 10 min respectively, and dried at room temperature. A 5% (w/w) Nafion-117 solution is diluted to 1% (w/w) with absolute ethanol. 5 μL of 1% (w/w) Nafion is dropped onto the SPE and dried at room temperature. The pretreated electrode is scanned by cyclic voltammetry in the 0.1 mol L^{-1} (pH 8.0) phosphate buffer solution (PBS) with 1 mmol L^{-1} MB at room temperature. Eventually, a Nafion–MB modified electrode is ready for use.

2.5. Preparation of alcohol biosensor

4 mg of ADH 5 mg of NAD^+ and 10 mg of BSA are dissolved in 200 μL of 0.1 mol L^{-1} PBS (pH 8.0), then 20 μL of 2.5% (w/w) glutaraldehyde solution is added to the mixed enzyme solution with vigorous stirring. 5 μL of the mixed solution described above is dropped onto the working area of the electrode and allowed to dry in air at room temperature for 12 h. The alcohol biosensor is prepared and kept dry in a refrigerator at 4 °C before use.

2.6. Analytical methods

All electrochemical measurements are carried out in a conventional three-electrode cell which is composed of saturated calomel reference electrode (SCE), a platinum wire counter electrode and a modified working electrode. Chromatographic analysis for serum alcohol concentration is performed according to the literature [30].

3. Results and discussion

3.1. Immobilization of MB onto Nafion

The immobilization of MB onto Nafion is carried out as described above, as shown in Fig. 2. With the scan proceeding, the anodic peak current (i_{pa}) and cathodic peak current (i_{pc}) rise

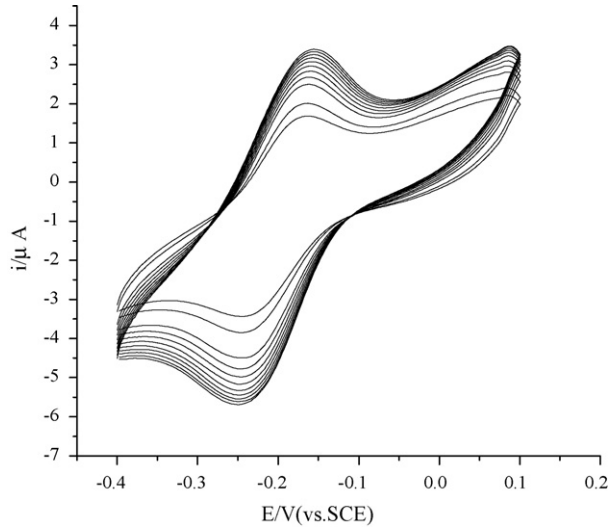

Fig. 2. Cyclic voltammograms of Nafion-modified SPE in 0.1 mol L^{-1} PBS (pH 8.0) with 1 mmol L^{-1} MB. Scan voltage, −0.4 to 0.1 V (vs. SCE). Scan rate: 50 mV s^{-1}. Temperature: 25 °C.

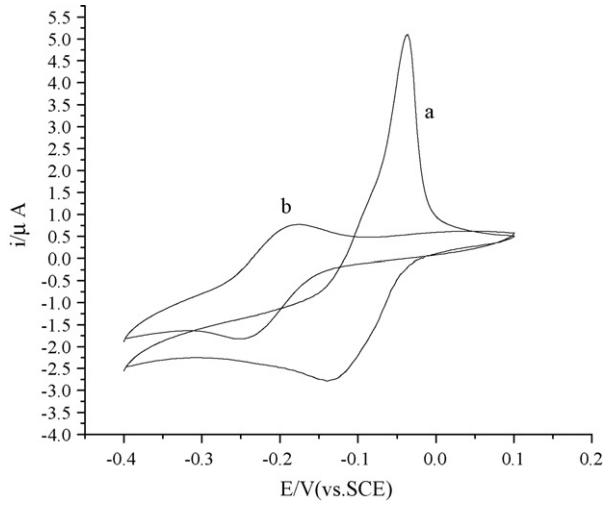

Fig. 3. Cyclic voltammograms of (a) a bare SPE in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} MB and (b) a Nafion–MB modified SPE in 0.1 mol L^{-1} PBS (pH 8.0). Scan voltage: −0.4 to 0.1 V (vs. SCE). Scan rate, 10 mV s^{-1}. Temperature, 25 °C.

Table 1
Stability of Nafion–MB modified SPE

pH	Absorbance (λ = 567 nm)
6.0	0
6.5	0
7.0	0
7.5	0
8.0	0
8.5	0

until they reach the steady state after a few minutes, which shows that MB could be absorbed by Nafion. The possible explanation is that MB is oxidized to MB^+ and Na^+ in the sulphonate group of Nafion is replaced by MB^+ [27].

Table 1 shows the stability of Nafion–MB modified SPE. After the six freshly prepared Nafion–MB modified SPE are respectively immersed in the 0.1 mol L^{-1} PBS during the pH range between 6.0 and 8.5 for 2 h at room temperature, Absorbance (λ = 567 nm) is monitored by a UV-265 ultraviolet-visible spectrophotometry. The results means that MB could be firmly absorbed by Nafion and adsorption could be independent on pH, possibly due to ion exchange between Nafion and MB.

3.2. Behavior of Nafion–MB modified SPE

Cyclic voltammetry is used to ascertain the electrochemical properties of the bare SPE and the Nafion–MB modified SPE. Fig. 3 are the cyclic voltammograms of (a) a bare SPE in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} MB and (b) a Nafion–MB modified SPE in 0.1 mol L^{-1} PBS. The sharper anodic peak and the smaller cathodic peak appear, as shown in Fig. 3a. The shape of the peak shows that the reduced form of MB could be adsorbed on the SPE and the oxidized form of MB could not be adsorbed on the SPE. The possible explanation is the different water solubility of the reduced and oxidized form of MB. However, in the voltammogram obtained for Nafion–MB modified SPE (Fig. 3b), the symmetrical anodic and cathodic peak is observed, which shows that a reversible redox reaction occurs in the electrode. The possible explanation is that MB could be immobilized onto SPE by Nafion and the oxidized form of MB could not enter the solution. In addition, both i_{pa} and i_{pc} of Nafion–MB modified SPE are the smaller than that of bare SPE. It is most likely that the electron transfer rate of Nafion–MB modified SPE in PBS is the slower than that of bare SPE in MB solution. The anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) both shift toward the negative value, when compared to that obtained for a bare SPE in MB solution. The possible explanation is that anions in PBS enter the MB–Nafion film to counterbalance the positively charged MB^+ and Na^+ in the film.

Fig. 4A shows the cyclic voltammograms of a Nafion–MB modified SPE, obtained in 0.1 mol L^{-1} PBS (pH 8.0) at the different scan rates in the scanning potential range (−0.4 V and +0.1 V vs. SCE). Both i_{pa} and i_{pc} rise continuously with scan rate and are linearly proportional to the square root of scan rate in the range from 10 to 100 mV s^{-1}. The ration of i_{pa} and i_{pc} is approximately one. In addition, E_{pa} and E_{pc} does not shift much with change of scan rate, the separation of peak potentials (ΔE_p) is less than 90 mV. These results suggest that the electrochemical reaction is the similar to the diffusion-controlled redox process. Fig. 4B shows the relationship between the square root of scan rate and the anodic peak current. (Linear regression equations, i_p: $y = -0.495 + 0.509x$, $r = 0.997$).

3.3. Current response of biosensors to alcohol

Fig. 5A presents alcohol biosensor mechanism of response. When alcohol is oxidized to acetaldehyde, NAD^+ is reduced to NADH under the catalysis of ADH and NAD^+ immobilized on the electrode. Meanwhile, MB could again electrocatalyze the oxidation of NADH to NAD^+, and electrons would be quickly transferred between enzymes and the modified working electrode so that the increase of the anodic current could be

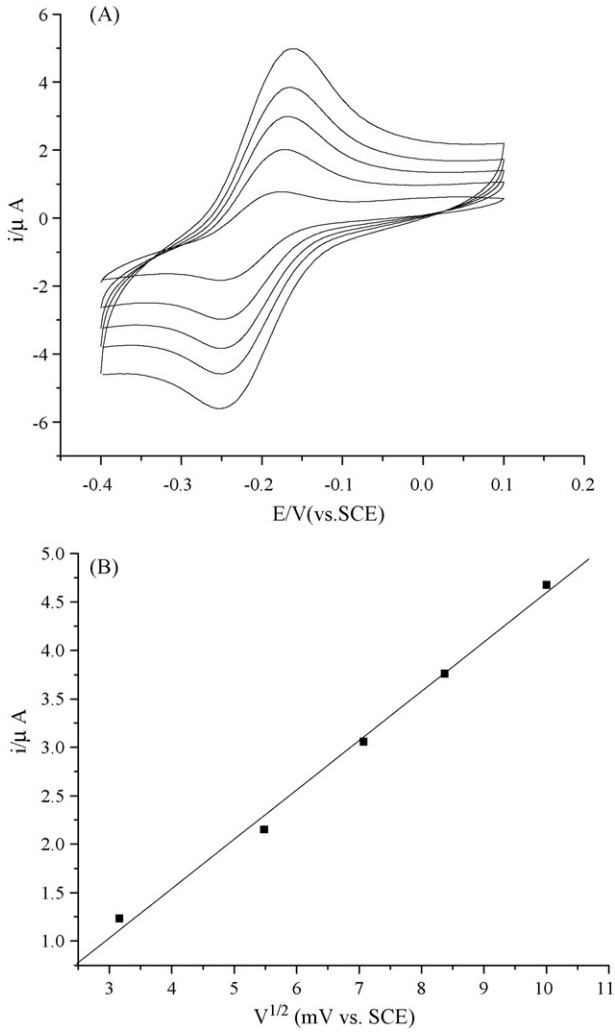

Fig. 4. (A) Cyclic voltammograms of a Nafion–MB modified SPE in 0.1 mol L^{-1} PBS (pH 8.0) with 1 mmol L^{-1} MB. Scan voltage: −0.4 to 0.1 V (vs. SCE). Scan rate: (10 mV s^{-1}), 10, 30, 50, 70, 100 (from inner to outer). Temperature: 25 °C. (B) Relationship between the square root of scan rate and the anodic peak current.

detected amperometrically. Fig. 5B shows cyclic voltammograms of the biosensor for the absence (a) and the presence (b) of 1 mmol L^{-1} alcohol in 0.1 mol L^{-1} PBS (pH 8.0) at a scan rate of 10 mV s^{-1}. As shown in Fig. 5B, the anodic current increase rapidly and the cathodic current decrease at the same time, which shows the catalytic reaction occurs on the biosensor through the mediated mechanism.

3.4. Optimization of the operating conditions

The working potential is investigated, since it could influence tremendously on the biosensor response for alcohol. As shown in Fig. 6, the amperometric response for alcohol starts about −0.45 V, and reaches a maximum current intensity at −0.17 V and keeps the plateau until −0.12 V (vs. SCE). Accordingly, −0.17 V versus SCE is chosen as the optimal working potential, because such a potential could obtain the higher current intensity and reduce the interference of easily oxidizable substances in the real samples.

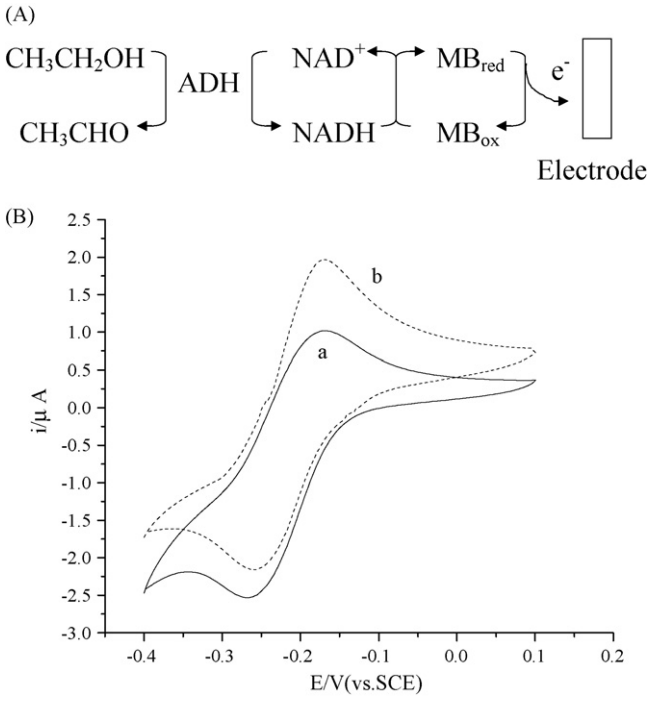

Fig. 5. A Alcohol biosensor mechanism of response. Med_{red}, reduced mediator Med_{ox}, oxidized mediator. (B) Cyclic voltammograms of the biosensor for the absence (a) and the presence (b) of 1 mmol L^{-1} alcohol in 0.1 mol L^{-1} PBS (pH 8.0). Scan voltage: −0.4 to 0.1 V (vs. SCE). Scan rate: 10 mV s^{-1}. Temperature: 25 °C.

The effect of pH on the biosensor response for alcohol is investigated during the pH range between 6.0 and 8.5 in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} alcohol. As shown in Table 2, the current intensity increase with the solution pH increasing from 6.0 to 8.0, reaching a maximum current at pH 8.0. The possible explanation is that some necessary groups of enzyme have the different dissociating power in the different pH condition and those could easily combine with substrate in the certain pH condition. Accordingly, this pH range reflects the

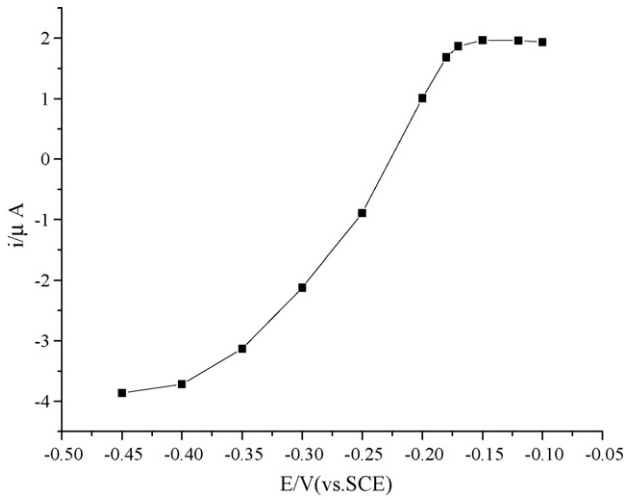

Fig. 6. Variation of biosensor response to 1 mmol L^{-1} alcohol with working potentials in 0.1 mol L^{-1} PBS (pH 8.0).

Table 2
Effect of buffer solution pH on the biosensor response for alcohol, obtained in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} alcohol at the working potential of −0.17 V (vs. SCE), at 25 °C ($n = 3$)

pH	Δi/nA
6.0	42.3 ± 0.6
6.5	55.8 ± 0.4
7.0	73.4 ± 0.5
7.5	82.3 ± 0.6
8.0	98.6 ± 0.3
8.5	88.7 ± 0.6

optimal conditions for both the enzymatic and electrochemical reactions in the biosensor.

The different buffer solutions with the same pH and concentration also influence on the biosensor response for alcohol. As shown in Table 3, the maximum current intensity is observed in the PBS. The possible explanation is the complex formation between NADH and phosphate, like an adduct, making the NADH oxidation easier [31]. Based on these results, the phosphate buffer solution is chosen as the optimal buffer solution.

The effect of temperature on the biosensor response for alcohol is demonstrated for the range between 10 and 40 °C. As shown in Fig. 7, the response current increases with the temperature. However, according to the properties of enzyme that higher temperature would bring about the partial denaturation of the enzyme, further increase of temperature would result in a decrease of the response current. Accordingly, 25 °C is selected as the operating temperature.

3.5. Analytical curve for alcohol

Fig. 8 shows a chronoamperometric response plots obtained from 1 to 5 mmol L^{-1} of alcohol in 0.1 mol L^{-1} PBS (pH 8.0) at the working potential of −0.17 V (vs. SCE), at 25 °C. The biosensor response time is very short, reaching 95% of the steady-state current within 30 s. The biosensor response time is excellent considering that it is a carbon electrode based on screen-printing technology and mediator immobilized on the electrode could quickly transfer the electrons between the enzyme and electrode. Fig. 8 (inset) shows a linear calibration curve of the steady-state current versus alcohol concentration. The analytical curve is calibrated by the linear regression equation: Δi (nA) = 13.24 + 126.8C (mmol L^{-1}), $r = 0.9895$. The detection limit of the biosensor is estimated to be 1.1×10^{-5} mol L^{-1} alcohol at a signal to noise ration of 3. The linear response range of the biosensor to alcohol can be

Table 3
Effect of buffer solution on the biosensor response for alcohol, obtained in 0.1 mol L^{-1} (pH 8.0) buffer solution with 1 mmol L^{-1} alcohol at the working potential of −0.17 V (vs. SCE), at 25 °C ($n = 3$)

Buffer solution	Δi/nA
Phosphate	98.6 ± 0.3
Tris	91.4 ± 0.5
KCL	64.6 ± 0.4

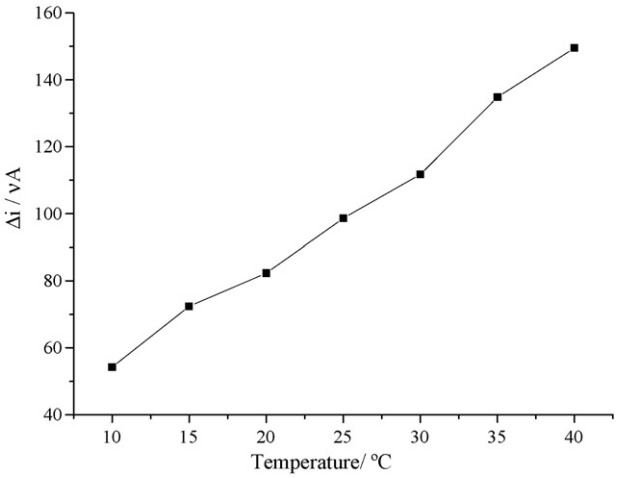

Fig. 7. Effect of temperature between 10 and 40 °C on biosensor response for alcohol, obtained in 0.1 mol L^{-1} PBS (pH 8.0) with 1 mmol L^{-1} alcohol at the working potential of −0.17 V(vs. SCE).

extended at least to 5 mmol L^{-1}. The apparent Michaelis–Menten constant (K'_m) can be estimated by the electrochemical version of the Lineweaver–Burk equation, $I_k = I_m - K'_m \times I_k/[S]$ [32]. Where I_k is the electrocatalytic current after the addition of the substrate [S] is the bulk concentration of the substrate, and I_m is the maximum current measured under saturate condition. A value of K'_m is calculated to be 5.8 mmol L^{-1}, which is the smaller than that reported in the literature [25], showing that immobilization procedure could not result in a significant change in the enzyme activity and the biosensor presents higher affinity to alcohol.

3.6. Interferences

The electroactive substances commonly present in the real serum samples, such as methanol, glucose, can cause problems in accurate determination of alcohol. The influence of these interferents on the biosensor response for alcohol are also

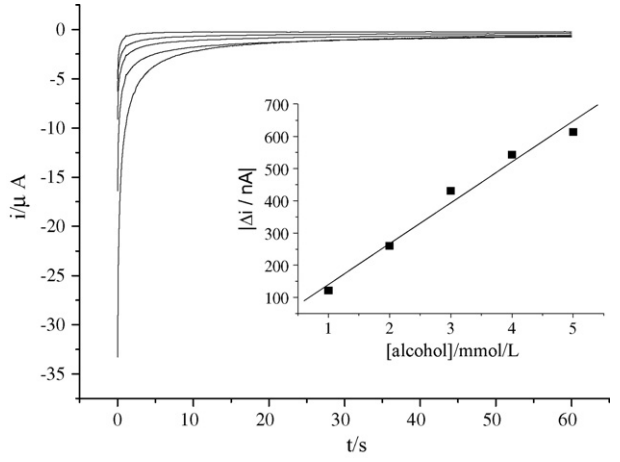

Fig. 8. Chronoamperometric response plots of biosensor obtained in 0.1 mol L^{-1} PBS (pH 8.0) at the working potential of −0.17 V (vs. SCE), at 25 °C. Alcohol concentration from top to bottom: 1, 2, 3, 4, and 5 mmol L^{-1}. Inset: calibration curve of biosensor as a function of alcohol concentration.

Table 4
Effect of interferents on the biosensor response for alcohol, obtained for the presence of interferent in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} alcohol at the working potential of −0.17 V (vs. SCE), at 25 °C ($n = 3$)

Interferents 1 mmol L^{-1}	Interference current (nA)
Methanol	5.3 ± 0.3
Glucose	2.8 ± 0.2
Lactic acid	3.6 ± 0.3
Ascorbic acid	2.1 ± 0.1

investigated in 0.1 mol L^{-1} PBS (pH 8.0) at the working potential of −0.17 V (vs. SCE), at 25 °C. As shown in Table 4, no distinguished interference current ($IC = I_2 - 1 - I_1$) is observed, where I_1 is the initial response current obtained in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} alcohol and I_2 is the response current obtained for the presence of 1 mmol L^{-1} interferent in 0.1 mol L^{-1} PBS with 1 mmol L^{-1} alcohol. These results show that the interference current can be ignored because the currents are too low to influence on the biosensor response for alcohol. Absence of sample interference can be related to the low working potential and the specificity of ADH. This is very important because it is unnecessary to make the differential measurements for alcohol determination.

3.7. Reproducibility and stability of the biosensor

The reproducibility of the identical biosensor is examined in 0.1 mol L^{-1} PBS (pH 8.0) with 1 mmol L^{-1} alcohol at the working potential of −0.17 V (vs. SCE), at 25 °C. The relative standard deviation is 3.6% for 10 successive assays. The batch reproducibility is the most important factor in the practical application of disposable biosensor. 5 electrodes are randomly selected from 5 batches of screen-printed electrodes to fabricate the alcohol biosensors independently. The fabrication reproducibility of 5 biosensors is examined in above solutions. The acceptable batch reproducibility with a relative standard deviation of 4.3% is obtained. The storage stability of biosensor toward the response for alcohol is examined as well. After being stored at 4 °C for 10, 20, 30 days, the response current decreased by 3.7%, 5.2%, 8.4% of the initial response, respectively. The above results indicate good reproducibility and stability of the biosensor.

3.8. Analysis of real serum samples

To illustrate feasibility of the biosensor in practical analysis, the biosensor is used to detect alcohol in human serum samples. Real serum samples of two drunk drivers were obtained from Chongqing institute of forensic medicine. After the samples are centrifuged, the clear supernatants are diluted appropriately and used without previous filtration or any other pretreatment. The samples are firstly analyzed by a GC-9A gas chromatography. Then the samples are assayed with biosensor. Table 5 shows the concentration found and the recoveries obtained by the biosensor. As observed, the data shows the good correlation between the results obtained with the biosensor and those obtained by gas chromatography. Moreover, the biosensor also presents good recovery values. This biosensor demonstrates accurate and selective determination of serum alcohol.

Table 5
Results of alcohol measurements in serum samples ($n = 3$)

Found in diluted serum (mmol L^{-1})[a]	Added (mmol L^{-1})	Total (mmol L^{-1})[b]	Recovery (%)
	0.2	0.79 ± 0.02	96.5 ± 2
0.62	0.4	1.02 ± 0.04	100.1 ± 4
0.4	0.6	1.21 ± 0.04	99.2 ± 4
1.02	0.2	1.14 ± 0.08	102.1 ± 8
0.91	0.4	1.36 ± 0.06	103.5 ± 6
	0.6	1.46 ± 0.05	96.7 ± 5

[a] Alcohol concentration determined by gas chromatography.
[b] Alcohol concentration determined by alcohol biosensor.

4. Conclusion

The main aim of the paper is to fabricate the disposable amperometric biosensor based on screen-printed electrode technology. The preparing procedure is simple, fast and economical. The experiments described above illustrate the capability of the biosensor for screening serum alcohol concentration, without adding any reagent. It shows good sensitivity, accuracy and speed which are required for any analytical methodologies. The proposed biosensor may provide a useful screening procedure for the determination of serum alcohol in intoxicated drivers. However, post-mortem analysis of blood alcohol appears not possible since it cannot differentiate between common alcohols and aldehydes found in postmortem specimens.

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